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Comparison of Rat Cone ERG Elicited by a Pulse Flicker and Sine-Wave Modulated Light Stimuli

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1. Introduction

The electroretinogram (ERG) affords a quantitative, objective, and noninvasive method by which to examine light-evoked neuronal activity, and is commonly used to study the functional integrity of normal and diseased retinas (Fishman, 2001; Frishman, 2006; Peachey and Ball, 2003). ERG elicited by a periodic stimulus (flicker ERG) is a useful tool to investigate temporal property of retinal circuitry, especially cone pathway in the retina (Alexander et al., 2005; Burns et al., 1992; Kondo and Sieving, 2001; Qian et al., 2010; Qian et al., 2008). Two kinds of light stimuli are commonly used for flicker ERG recordings: sine-wave modulated light and a pulse light flickers. Sine-wave modulated light, due to its simplicity in spectrum component, is often used to characterize frequency-response relationship of ERG (Alexander et al., 2003; Burns et al., 1992; Kondo and Sieving, 2001; Krishna et al., 2002; Qian et al., 2008; Viswanathan et al., 2002). On the other hand, pulse light flicker can easily be implemented technically, and has long been applied for clinical use (Audo et al., 2008; Garry et al., 2010; Jacobs et al., 1996). Indeed, 30 Hz pulse flicker under light-adapted condition is recommended by the International Society for Clinical Electrophysiology of Vision (ISCEV) for evaluating activity of the cone system in human patients (Marmor et al., 2009).

For a pulse train consisting of a series of flashes with intensity I , duration d , and period P , the Fourier transform in the frequency domain is given by the expression:

$$x(t) = I \left[k + \frac{2}{\pi} (\sin k\pi \cos \omega t + \frac{1}{2} \sin 2k\pi \cos 2\omega t + \dots + \frac{1}{n} \sin nk\pi \cos n\omega t + \dots) \right]$$

where $k = d/P$ and $\omega = 2\pi/P$. When the duration of each flash is very brief (i.e. d is small compared to P , such as is with a xenon flash), k is small and $\sin(nk\pi)$ approaches $nk\pi$. Under such condition, the Fourier transform of a pulse flicker reduces to:

$$x(t) = I [k + 2k(\cos \omega t + \cos 2\omega t + \dots + \cos n\omega t + \dots)]$$

Therefore, for a flickering stimulus comprised of a series of brief pulses, the frequency domain consists of a series of harmonics, each with equal energy. In other words, pulse

flicker can be viewed as a mixture of sine-wave flicker at fundamental and harmonic frequencies. Consequently, for a linear system, the frequency-response relation derived from harmonic components of the ERG response to one pulse train flicker should be similar as those derived with a series of sine-wave modulated light stimuli. In this study, we recorded rat cone flicker ERG elicited by these two light stimuli from the same animals and compared the response in the frequency domain derived from Fourier analysis (Bach and Meigen, 1999).

Our results indicate that, for response frequencies less than 30 Hz, frequency-response relationship derived with a single pulse flicker stimulus has similar shape as the one derived from the fundamental responses to a series of sine-wave light stimuli. Therefore, pulse flicker ERG can be used as an alternative method to probe the frequency-response relationship of flicker ERG at low response frequencies.

On the other hand, the high frequency harmonic responses derived from a pulse flicker ERG consistently contained higher peaks than the fundamental response of sine-wave flickers. In this study, we investigated two potential mechanisms for these high harmonic components: a nonlinear response to harmonic components in a pulse flicker and ERG oscillatory potentials.

Oscillatory potentials (OPs) are wavelets that superimposed on the rising phase of the ERG b-wave. OPs have been shown to arise within the proximal retina (Wachtmeister and Dowling, 1978), and the individual peaks have different retinal depth profiles, suggestive of distinct cellular origins (Wachtmeister, 1980). The specific retinal cells that are responsible for the OPs are still being debated, but there is strong evidence that GABAergic neurons, and their synaptic interactions, are key elements in the generation of the response (Wachtmeister, 1980). Bicuculline, an inhibitor for GABA_A receptor activity in the retina, reduces OP in flash ERG (Wachtmeister, 1980). In this study, we used bicuculline to probe the contribution of OPs in high harmonic responses of ERG elicited by pulse flicker stimulus.

In a non-linear system, response to a mixture of two sine-wave stimuli will not only include fundamental responses at each stimulus frequencies, it also includes components (beats) at both the difference and the sum of these two stimulus frequencies and their multiples. These beats responses have been used to investigate nonlinearities in human flicker ERG (Alexander et al., 2001; Burns et al., 1992; Wu et al., 1995). In this study, we used a beat response to measure the non-linearity in rat flicker ERG response. Our results indicate that nonlinearity of retinal signal processing mechanisms in rat eye also has a large contribution to the high harmonic responses observed with ERG responses elicited by pulse light stimuli.

2. Methods

2.1 Animals and anesthesia

Adult pigmented (Long Evans) rats (both sexes, weight 250-500g) were used for this study. Animals were housed in the Biological Resources Laboratory (BRL) of the University of Illinois at Chicago in a 14/10-hour light/dark cycle, the standard lighting regimen of the BRL. Rats were anesthetized with ketamine (100 mg/kg) and xylazine (6 mg/kg), and the pupils were dilated with topical phenylephrine (2.5%) and tropicamide (1%). Topical proparacaine (0.5%) was used to anesthetize cornea. Body temperature was maintained at ~37°C with a heating pad. All experimental procedures conformed to the statement on animal care of the Association for Research in Vision and Ophthalmology.

2.2 ERG recording and light stimulation

Instrumentation and recording procedures have described in our previous publication (Qian et al., 2008). Light stimuli were delivered by multiple light-emitting diodes (LEDs) with a peak wavelength of 505 nm (Nichia NSPE590S, Tokushima, Japan) mounted in a small integrating sphere (Oriel 70500, Newport Corp., Stratford, CT) to provide a full-field stimulus. The current driving the LEDs was pulse-width modulated at 1 MHz under computer control. Pulse flickers were composed of ≤ 4 ms flashes delivered at various temporal frequencies (2 to 12 Hz). In most experiments, the peak luminance of each pulse was 690 cd/m² unless otherwise specified. A background light (5 cd/m²) was used to saturate the rod pathway (Xu et al., 2003). Eyes were adapted to background luminance before ERG recording. Sine-wave modulated light stimuli had a mean luminance of 100 cd/m² and Michelson contrast of 90%. Each stimulus was about 10s long, and contained an even number of cycles (or pulses). For mixture sine-wave stimulus, light intensity was modulated by sum of two sine-waves, each has a contrast of 45% and period of 56 ms (18 Hz) and 42 ms (24 Hz), respectively. ERG responses were recorded from a chlorided silver wire electrode placed in the centre of the cornea and connected to the input stage of a Grass AC amplifier (Model P511, with a bandwidth of 0.3 to 300 Hz and without a 60 Hz notch filter) and a sampling frequency of 2 kHz.

2.3 Intravitreal injection

Bicuculline methchloride (Sigma-Aldrich, St. Louis, MO) was dissolved in mammalian saline solution and delivered to the anesthetized rat eye by intravitreal injection through a glass capillary needle introduced into the vitreous cavity by piercing the sclera 3 mm posterior to the temporal limbus at approximately a 45-degree angle to the optical axis. The injection site was monitored under a dissecting microscope and a 3- μ l aliquot of a solution containing 1 mM bicuculline was injected into each eye. The final vitreal concentration (~ 80 μ M) was derived by assuming complete mixing in the rat vitreous with an estimated volume of 38 μ l (Xu et al., 2003).

2.4 Data analysis

The amplitudes of each harmonic component were derived from discrete Fourier transforms using the Matlab Signal Processing Toolbox (The Mathworks, Boston, MA). For each 10s recordings of ERG waveform, about 500 ms of data at the beginning and end of the recording were omitted. The exact length that was omitted depended on the stimulus period and was an even number of cycles or pulses. As a result, Fourier analyses of ERG were based on approximately 9-s segments of continuous data, consisting of an even number of cycles or pulses. We adopted a criterion for harmonic component responses as greater than 3 times the noise level at neighboring frequencies, which exceeds the 5% significant level (Meigen and Bach, 1999). A digital zero-phase band-pass filter (40-200 Hz) was used to isolate the OP components from flash ERG (Ramsey et al., 2006). Data analysis tools in Microsoft Excel were used to calculate statistical significance (two-tail *t*-test analysis).

3. Results

3.1 Frequency-response relationship for sine-wave flicker ERG and pulse flicker ERG

Fig. 1A illustrates flicker ERG responses elicited from a rat eye by sine-wave modulated light stimuli at selected temporal frequencies with 90% contrast. For clear presentation, the

traces on right side (responses to high frequency stimuli) are magnified 5 times more than those on the left side (responses to low frequency stimuli) of the figure. [Examples of rat flicker ERG responses elicited by sine-wave stimuli plotted on the same magnitude scale can be find in Figure 1 of (Qian et al., 2008)]. For presentation purpose, each waveform shown in the figure represents an averaged 500-ms segment over 10-s recorded responses. The top panel of Fig. 1B shows an example of flicker ERG response elicited from the same rat eye by a 6-Hz pulse flicker light. The trace represents an averaged 1-s segment over 10-s recorded responses, and is plotted with the same scale as those shown on the left side traces of Fig. 1A. Fourier spectrum of the entire 9-s ERG waveform is shown in the bottom panel of Fig. 1B. It is clear that, in addition to fundamental response at 6 Hz, there are prominent harmonic components in rat pulse flicker ERG response.

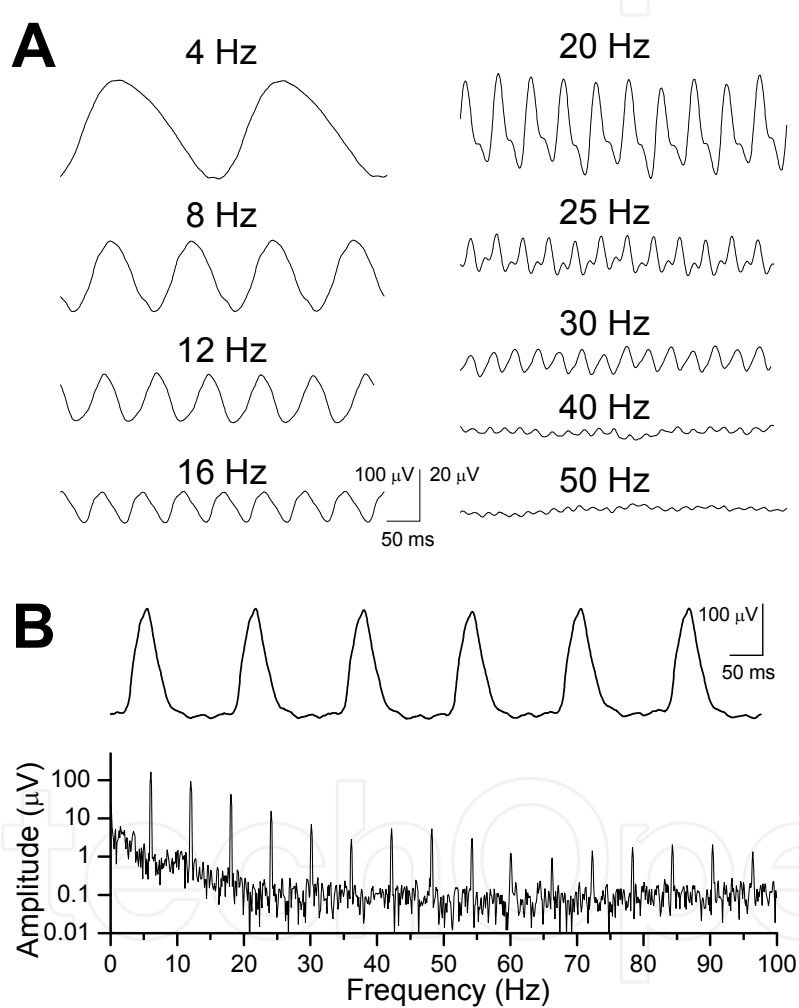


Fig. 1. Examples of typical ERG waveforms elicited from a rat eye. **A.** ERG waveforms elicited by sinusoidally modulated light stimuli at 90% contrast presented as selected temporal frequencies indicated above each traces. Each trace represents the average of eighteen 500-ms segments from a 10-s recording. Please note the traces on right side were plotted with 5 x more amplification than those on the left. **B.** Top, ERG waveform elicited by 6 Hz pulse flicker from the same eye as in **A.** An averaged 1-s segment was shown for clarity. Bottom, Fourier spectrum of the 9-s ERG trace of pulse flicker ERG. In addition to fundamental response at 6 Hz, other harmonics are clearly evident.

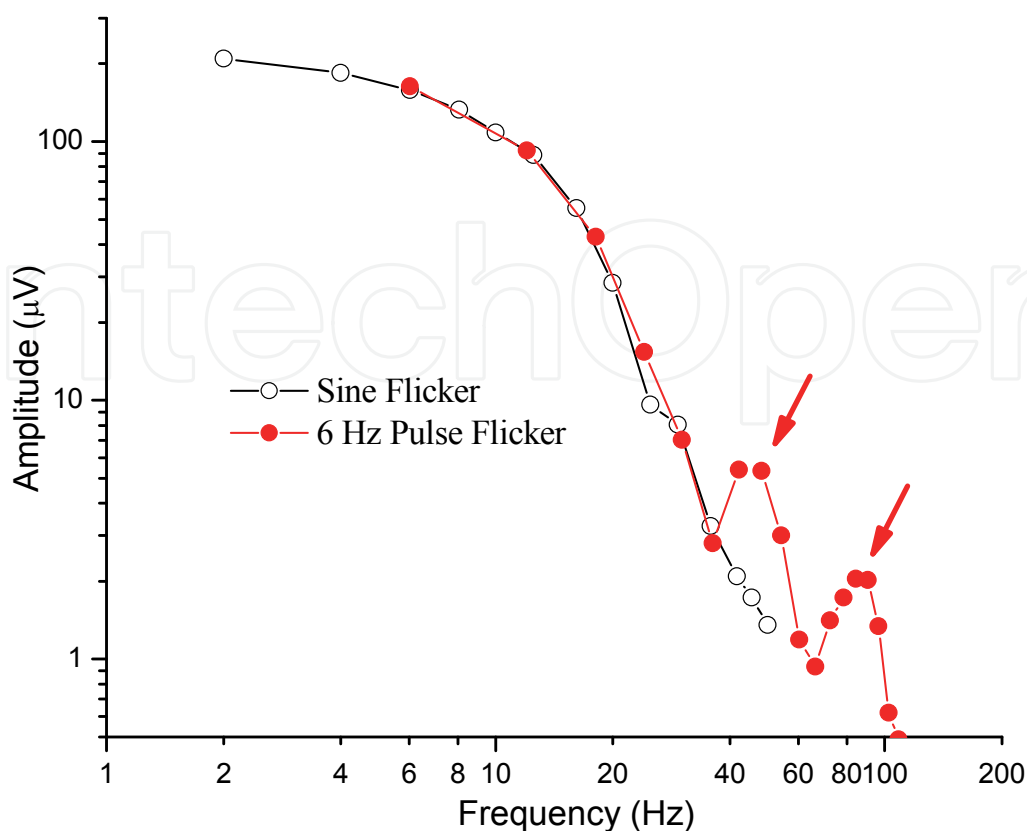


Fig. 2. Frequency-response relationship of rat ERG elicited by sine-wave flickers and pulse flickers. The amplitudes of the harmonic components of pulse flicker at 6 Hz and the fundamental responses of sine-wave flicker at various temporal frequencies are plotted. For response frequencies <30 Hz, frequency-response relation for pulse flicker ERG exhibits a shape similar to that derived for sine-wave flicker ERG. For response frequencies >30 Hz, larger peaks are observed in pulse flicker ERG than sine-wave ERG responses (arrows).

Fig. 2 shows frequency-response relationship of rat cone ERG derived by Fourier analysis of the waveforms shown in Fig. 1. For the response elicited by sine-wave modulated light stimuli, the amplitudes of the fundamental responses at each respective stimulus frequency are plotted. In agreement with our previous report (Qian et al., 2008), the amplitude of the fundamental response to sine-wave flicker exhibited a monotonic low-pass pattern. Response elicited by a 6 Hz pulse flicker is plotted as the amplitudes of fundamental and harmonic components. For response frequencies <30 Hz, the amplitudes of fundamental and harmonic responses to pulse flicker exhibit similar values as the fundamental response to sine-wave modulated light stimulus at respective temporal frequency. At high response frequencies, however, the amplitudes of harmonic responses to pulse flicker stimulus exhibit larger peaks than the fundamental response derived from sine-wave stimulus (arrows).

These high frequency harmonic components are common in rat cone ERG elicited with pulse flickers. Figure 3 shows averaged data from 12 eyes of the responses elicited by pulse flickers at frequency range from 2 to 12 Hz (open circles), and in comparison with the responses elicited by sine-wave flickers from the same animals (solid circles). The response amplitudes elicited by pulse flicker were smaller for lower temporal frequency stimulus

than those elicited by higher temporal frequency stimulus, indicating the dependence of response on stimulus energy since high frequency pulse flickers contain more energy for each harmonic component than low frequency stimulus. In all cases, a peak in the high harmonic component is evident at all flash frequencies tested (2-12 Hz, arrows). However, location of this high frequency component varied with the pulse stimulus frequency, i.e. the component appeared at higher response frequency with higher pulse flicker frequency.

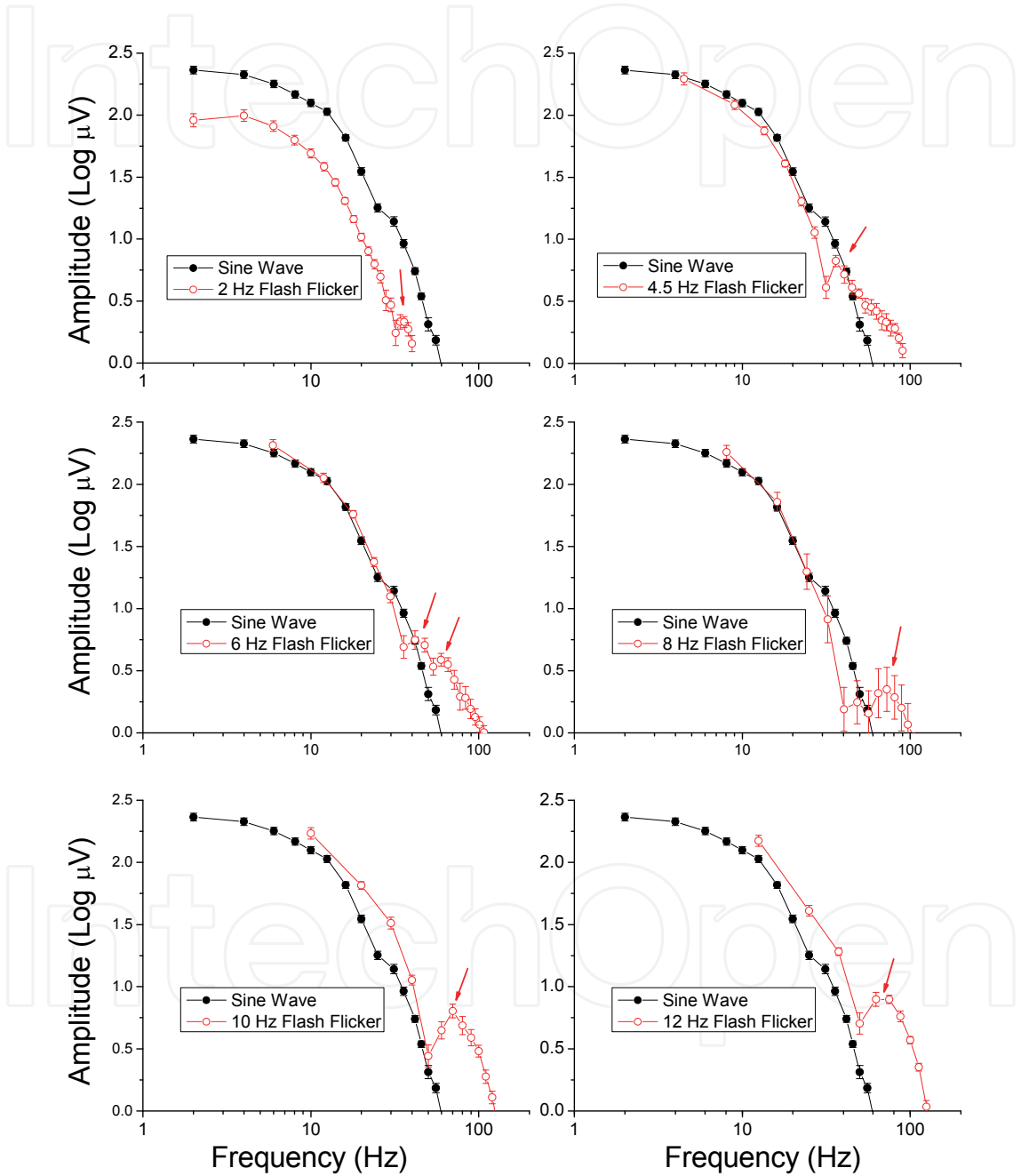


Fig. 3. Comparison of the mean frequency-response relationship elicited by sine-wave flicker ERG and with harmonic responses to pulse flicker ERG at various temporal frequencies. For all ERG responses elicited by pulse flickers, a peak in the high harmonic component is evident at all flash frequencies tested (2-12 Hz, arrows). Date was averaged from 12 eyes and presented as mean + SEM.

3.2 Effects of flash intensity on pulse flicker ERG

We examined the effects of flash intensity on frequency-response relationship of pulse flicker ERG elicited from rat eyes. Fig. 4 shows the results, averaged from 8 rat eyes, of frequency spectrum of ERG responses elicited with 6-Hz pulse flickers when the flash intensity varied from 50 cd/m² to 690 cd/m². In all flash luminosities tested, humps in high harmonic components were observed in pulse flicker ERG responses. It has been reported that, for sine-wave flicker ERG, responses elicited from rat eyes exhibit two distinct processes at low and high temporal frequencies (Qian et al., 2008). In particular, the contrast-response relation is linear when tested with a low-frequency (6 Hz) stimulus, but saturated in response to a high-frequency (20 Hz) stimulus. We tested if these two distinct retinal processes could also be revealed with pulse flicker stimulus. Fig. 5 plotted intensity-response relationship of 6-Hz pulse flicker for fundamental response (6 Hz) and fourth harmonic (24 Hz). Data were normalized to response elicited with maximal flash intensity (690 cd/m²). Similar as observed with sine-wave stimulus, low frequency response (6 Hz) exhibited relative linear relationship in intensity-response function, whereas high frequency response (24 Hz) showed saturating behaviour. These results indicate that pulse flicker ERG can also be used to distinguish two retinal processes in rat eyes.

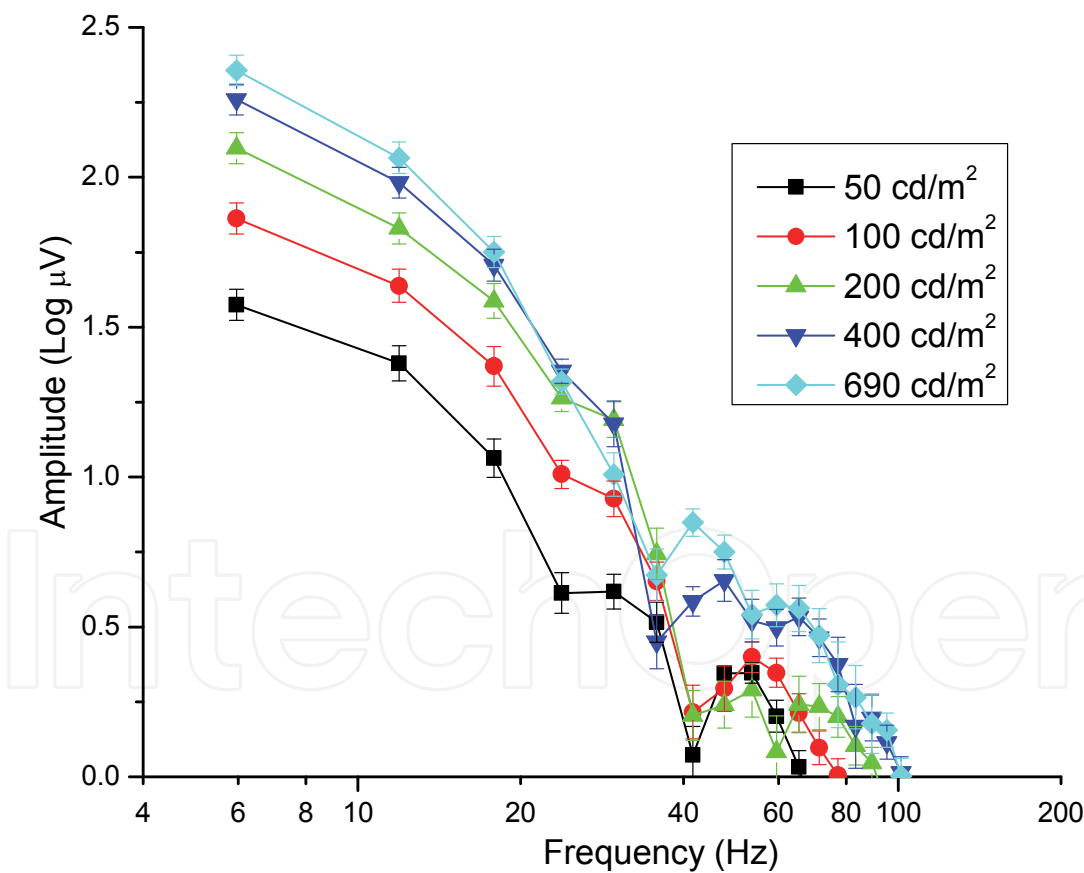


Fig. 4. Effects of flash intensity on the frequency-response relationship of rat pulse flicker ERG. Responses were elicited with 6-Hz pulse flickers. Flash intensity was varied from 50 cd/m² to 690 cd/m². Peaks in high harmonic components were observed at all flash intensities. Data was averaged from 8 rat eyes.

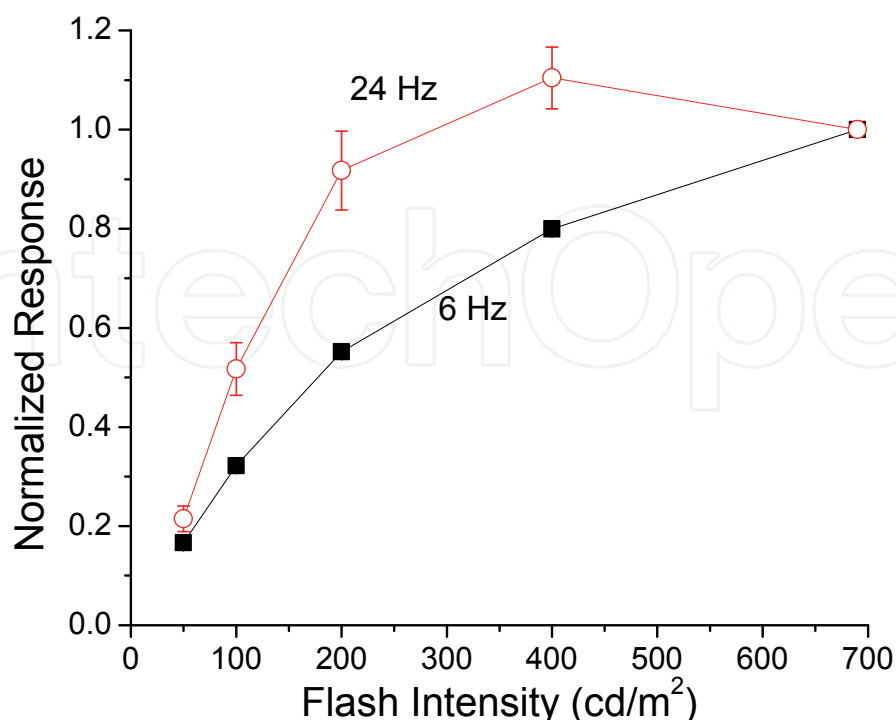


Fig. 5. Intensity-response relationship for fundamental (6 Hz) and fourth harmonic (24 Hz) of a 6 Hz pulse flicker ERG. Whereas the low frequency (6 Hz) response exhibited relative linear relation with the flash intensity, the high frequency response (24 Hz) reached saturation around 200 cd/m². Data were derived from those shown in Fig. 4.

3.3 Potential mechanisms for high frequency components in pulse flicker ERG

To examine retinal mechanism of the high frequency components in rat pulse flicker ERG, we isolated high frequency response waveform using a digital filter. An example is shown in Fig. 6. The top panel illustrates original response waveform for a 4.5 Hz pulse flicker ERG. The high frequency component isolated by a 40 Hz high-pass filter is shown at the bottom. It is clear that the high frequency components in pulse flicker ERG from rat eye mainly occurs at the rising phase of flicker ERG.

It is well known that oscillatory potentials (OPs) appear as the wavelets on the rising phase of flash ERG b-wave (Wachtmeister, 1980; Wachtmeister and Dowling, 1978). Therefore, it is possible that the high frequency components of rat pulse flicker ERG are mediated by OPs. OPs are generated in the inner retina by GABAergic neurons, most likely amacrine cells, and these ERG wavelets can be reduced by inhibiting GABA_A receptor activity in the retina (Wachtmeister, 2001). We examined the effect of intravitreal injection of bicuculline, a GABA_A receptor antagonist, on OPs of dark-adapted rat ERG elicited by a flash of light. Fig. 7 shows an example of the waveforms of flash ERG recorded from a saline injected and a bicuculline (80 μM) injected rat eye. The flash ERG waveforms are shown on top row, and bottom panel illustrates OPs isolated by a digital filter with bandwidth of 40 – 200 Hz. It is clear that bicuculline greatly reduced OP amplitudes in the rat eye.

We examined the effects of intravitreal injection of bicuculline on high frequency components of rat pulse flicker ERG, and results are shown in the left panel of bar graph in

Fig. 8. The high frequency components were derived by sum of harmonic components with frequencies higher than 40 Hz, and were normalized to the total flicker ERG response. When compared with responses elicited from saline injected eyes, the high frequency components from bicuculline-injected eyes were reduced by about 42% ($n=8$, $p<0.05$).

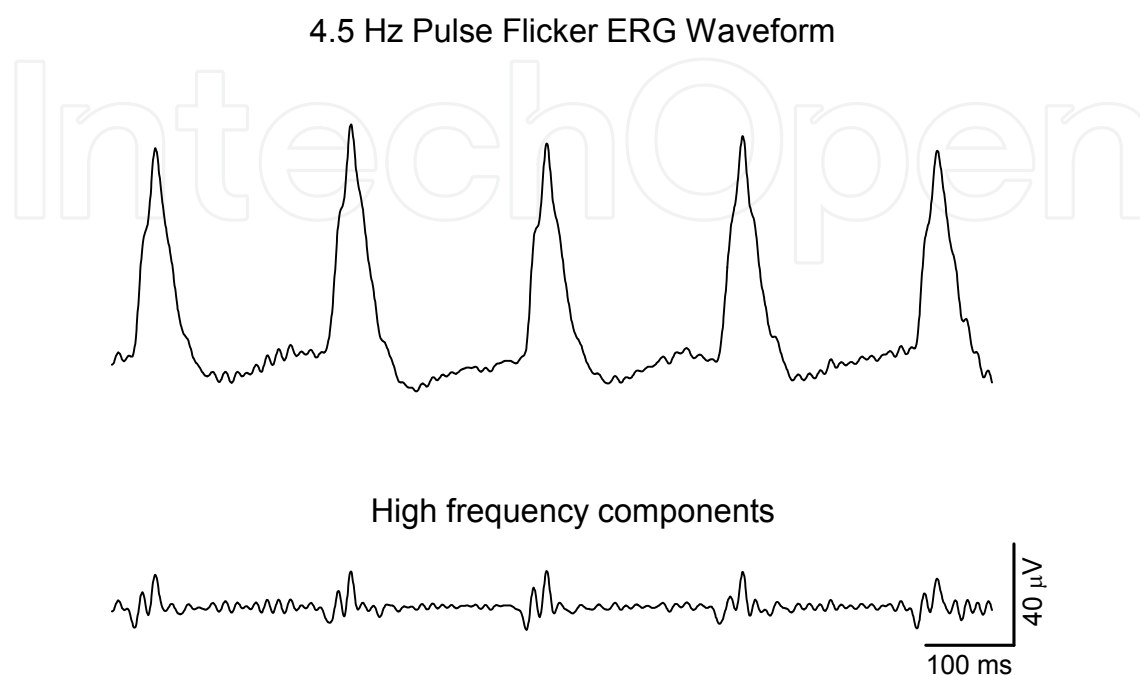


Fig. 6. Waveform of ERG elicited by 4.5-Hz pulse flicker. The original response waveform is shown at the top panel. The high frequency component (>40 Hz) of the response, derived with a high-pass digital filter, is shown at the low panel. The high frequency components occur at the rising phase of flicker ERG.

Another possible mechanism for generating high frequency components in pulse flicker ERG is the non-linear property of rat ERG responses. As pulse flicker stimulus itself contains multiple harmonic components each with similar energy strength, a non-linear response system will be able to generate beat components at high frequencies in response to low frequency stimulus. Such non-linear mechanism could also contribute to the observed high frequency components in pulse flicker rat ERG. To test this possibility, we examined non-linear responses by monitoring beat components of rat cone flicker ERG in response to a light stimulus that is composed of a mixture of two sine-waves, with frequency at 18 and 24 Hz respectively. Fig. 9 shows an example of frequency spectrum of the ERG response elicited from a rat eye by such light stimulus. The fundamental components for two stimuli frequency are pointed by arrows and marked as F1 and F2, respectively. In addition, beat components are also prominent in the response spectrum, as pointed by dashed arrows, indicating non-linear nature of rat cone flicker ERG response. To quantitate the amount of non-linearity in the system, we measured the ratio of the response at beat frequency of $F1 + F2$ to the sum of fundamental responses (i.e. response at frequency F1 and at F2). The results are summarized in the right panel of bar graph of Fig. 8. The bar graph also compared the effects of intravitreal injection of bicuculline on non-linear responses of rat cone flicker ERG. Interestingly, blocking GABA_A receptor in the retina with bicuculline increased non-linearity of the flicker ERG response, as measured by the beat component of harmonic components ($p<0.05$).

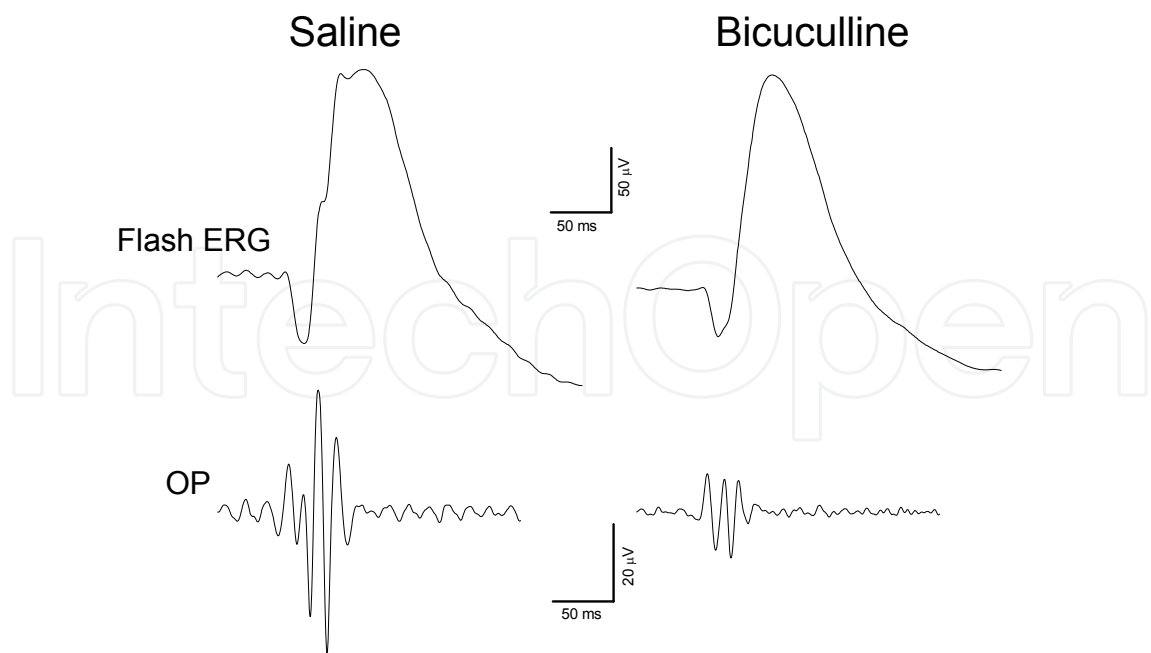


Fig. 7. Oscillatory potential (OP) in rat ERG. OPs (low panel) were isolated by a band pass filter (40-200 Hz) from the flash ERG illustrated in the top panel. Intravitreal injection of bicuculline greatly suppressed the OP amplitude with minimal effects on b-wave amplitude.

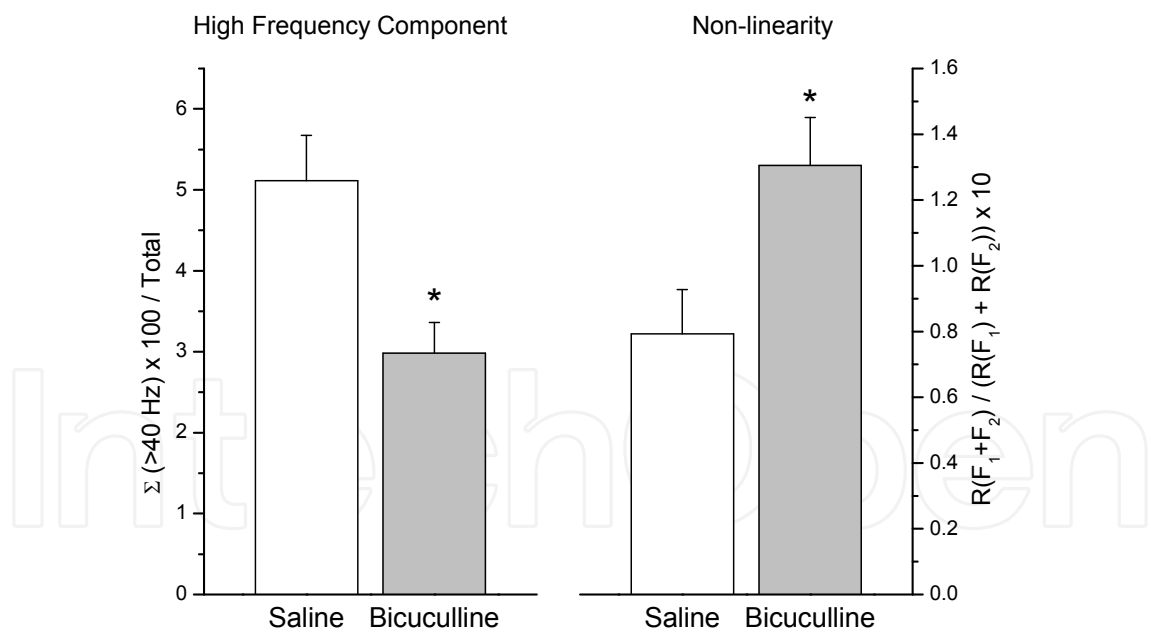


Fig. 8. Effects of bicuculline on high harmonic component and non-linearity in rat flicker ERG. A ratio of summed amplitude for harmonic responses above and below 40 Hz was used as an index for the high frequency component of pulse flicker ERG. Non-linearity was expressed as the ratio of beat response amplitude (F1 + F2) to the sum of the fundamental response to F1 (period 56 ms) and F2 (period 42 ms). Intravitreal injection of bicuculline (20 mM) significantly reduced the high frequency component in ERG elicited by pulse flicker, but enhanced the non-linearity of the response (*, $p < 0.05$). Data was averaged from 8 rats.

4. Discussion

In this study, we compared the frequency-response relationship for rat cone ERG elicited by flickering light stimuli, either to a series of flash pulses or to sinusoidally modulated light. Our results indicate that the frequency-response relationship derived from fundamental and harmonic component of pulse flicker ERG responses, with any temporal frequency range from 2 to 12 Hz, exhibited a similar shape as the fundamental response elicited by sine-wave flickers for response frequencies less than 30 Hz (Fig. 2 and 3). This is consistent with earlier observation that rat cone flicker ERG contains only small non-linear (second harmonic) components elicited with sine-wave stimulus for temporal frequency up to 16 Hz (Qian et al., 2008). Therefore, it is possible to use pulse flicker to determine low-pass character of rat cone flicker ERG. For practical purpose, a low pulse flicker will be a better choice since more harmonic components are available to provide response data points (Fig. 3). However, the exact amplitudes at each response frequency varied with the temporal frequency of pulse flicker stimulus (Fig. 3). This is large due to the fact that high frequency pulse flicker delivered more photons than low frequency stimulus, and the amplitudes of rat flicker ERG vary with flash intensity as shown in Fig. 4.

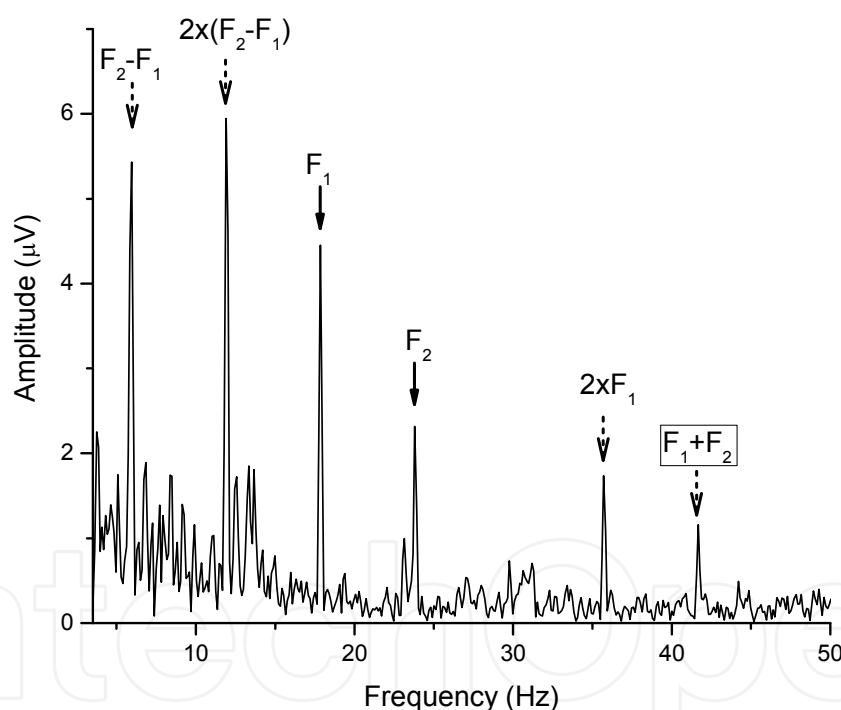


Fig. 9. Non-linearity in rat flicker ERG. Spectrum of ERG elicited by a mixture of two sine-wave modulated light stimuli with periods of 56 ms (18 Hz, F_1) and 42 ms (24 Hz, F_2), respectively. Fundamental responses to F_1 and F_2 are marked by arrows, beat components are marked by dashed arrows, and the beat response to $(F_1 + F_2)$ used as an index for non-linearity in rat flicker ERG was marked by a box.

On the other hand, it has been reported that primate photopic 32-Hz ERG for Sine-, square-, and pulsed stimuli were largely different, perhaps due to non-linear interactions among retinal signal pathways (Kondo and Sieving, 2002). It should be noted that rodent photopic ERG exhibit a number of difference from primates and diurnal animals (Alexander et al.,

2005; Ekesten et al., 1998; Hare and Ton, 2002; Qian et al., 2010; Rosolen et al., 2005; Shah et al., 2010). The ERG protocol developed for rodent eye may not be applicable to study of human ERG responses.

Using frequency-response relationship derived from harmonic analysis of pulse flicker ERG, we can also differentiate two distinct retinal processing mechanisms at low and high response frequencies (Fig. 4 and 5). Similar as responses elicited by sine-wave flickers (Qian et al., 2008), low frequency responses (fundamental response to 6 Hz pulse flicker stimulus) were relatively linear in relation with stimulus light intensity (Fig. 5), whereas high frequency responses (fourth harmonic responses to 6 Hz stimulus) have a relatively higher gain to low stimulus light intensity and responses become saturated with intensity above 200 cd/m². Therefore, pulse flicker stimulus will be particular valuable in cases that long-term recordings are hard to obtain, such as single cell recordings of retinal neurons. Instead of requiring multiple episodes of sine-wave modulated light stimuli, the responses elicited by a single pulse flicker stimulus will be enough to provide valuable information about frequency-response relationship for rodent eye with response frequency less than 30 Hz.

It should be noted that pulse flicker ERG does not capture all the features revealed by sine-wave flicker, which examines responses one temporal frequency at a time. For example, it is clear from response waveform shown in Fig. 1A that the flicker ERG elicited from this rat eye contained period-doubling for 20 Hz sine-wave frequency stimulus (Shah et al., 2010). However, this feature is not captured on frequency-response relationship derived from pulse flicker ERG (Fig. 1 and 2).

For response frequencies over 30 Hz, there were larger high harmonic components in pulse flicker ERG compared with the fundamental response elicited by sine-wave stimulus at corresponding frequency (Fig. 2 – 4). These high harmonic components in pulse flicker ERG were present for all flash frequencies tested (2-12 Hz) (Fig. 3), and persisted with the flash luminance varied from 50 cd/m² to 690 cd/m² (Fig. 4). To investigate the origin of these high harmonic components, we tested two possibilities: oscillatory potentials and non-linearity of the ERG response. The waveform of high frequency components is mainly located on the rising phase of the flicker ERG response (Fig. 6), a feature similar as oscillatory potentials of dark-adapted flash ERG. Intravitreal injection of a GABA_A receptor antagonist, bicuculline, both reduced oscillatory potentials in flash ERG and the high frequency components in pulse flicker ERG response (Fig. 7 and 8), indicating mechanisms similar as OP generators are contributing, at least in part, to the high harmonic components observed in pulse flicker ERG. To investigate the contribution of non-linearity in rat flicker ERG, we used a sum of two sine-wave modulate light at frequency of 18 and 24 Hz as stimulus, and measured beat response at frequency of 42 Hz, i.e. sum of 18 and 24 Hz. Our results indicate that non-linearity is a prominent component of rat cone flicker ERG in this region of response frequency (Fig. 9). Interestingly, eyes injected with bicuculline exhibited higher non-linearity compared with those of saline-injected eyes. The mechanisms of this action of GABA_A receptor antagonist are yet to be determined.

5. Conclusion

Pulsed light stimulus is commonly used to elicit flicker ERG, whereas sine-wave modulated light provides better characterization of frequency-response relation. In this study, we compared rat cone flicker ERG elicited by these two stimuli in frequency domain, and investigated the mechanism for the higher harmonic responses. Our results indicate that a

single pulse light stimulus can be used as an alternative method to probe the frequency-response relationship of flicker ERG for response frequencies less than 30 Hz. We also demonstrated that pulse light stimuli are useful to distinct two retinal processes revealed by sine-wave stimuli (Qian et al., 2008). In addition, pulse flicker ERG contains high harmonic components which have contributions from retinal generators of oscillatory potentials and non-linear interactions of retinal signal pathways. Pulse flicker stimulus could also be used as a tool to investigate retinal mechanisms of these two processes.

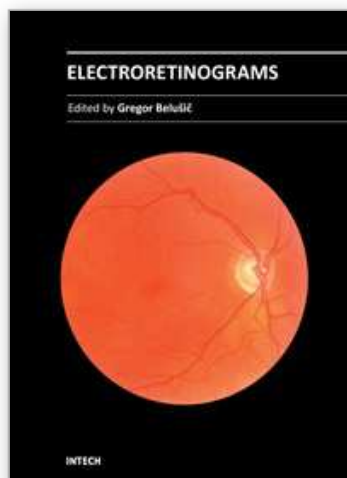
6. Acknowledgment

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